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Chapter VI

Lipid Rafts and Signal Transduction in Mast Cells

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Abstract

In mast cells, the first signaling process convincingly shown to involve lipid rafts was immunoglobulin E (IgE) signaling during an allergic response. The cross-linking of IgE bound to the high affinity IgE receptor (FcεRI), mediated by oligomeric antigen, is one of the key pathways in mast cell activation. Activation of FcεRI stimulates the release of numerous chemical mediators involved in allergic reactions.

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Mast cells, therefore, are a major source of cytokines and chemokines, similar to other immunoregulatory cells. The cross-linking of FcεRI, activates a transmembrane signaling process and leads to its translocation to specialized membrane micro-domains, known as lipid rafts. Together with FcεRI, other proteins, which are important for the signaling cascade in mast cells, are recruited to these micro-domains. Besides this recruitment, some transmembrane proteins, proteins with lipidic modification, high levels of cholesterol, and gangliosides are also present and support the formation of the signaling platform. Many cellular functions have been connected with lipid rafts including endocytosis, cholesterol transport, as well as signal transduction. Gangliosides are a characteristic component of lipid rafts and have been shown to act in cell-cell recognition, cell-substrate interaction, as receptors for virus and bacterial toxins, and also to have a role in proliferation, differentiation, adhesion, immune response, signal transduction. This chapter provides an overview of the importance of the lipid rafts in signal transduction in mast cells via FcεRI and the importance of lipid rafts in modulating biological processes induced by signaling in these cells.

Introduction

The fluid-mosaic model of cell membranes [1] postulated a uniform double layer of lipids with proteins randomly suspended in the bilayer. This lipid-protein bilayer delimits cells and organelles and acts as a barrier to the passage of polar molecules and ions [2-5].The model predicted free rotational and lateral diffusion of proteins and lipids within the plane of the membrane resulting in their random distribution. A number of lines of evidence indicating a non-random distribution of complex lipids and proteins in cell membranes was presented in seventies and eighties suggested that the fluid-mosaic model was probably inaccurate. Lee and collaborators [6], studying the effects of temperature on membrane behavior, proposed the presence of clusters of lipids in membranes. Wunderlich and collaborators [7] suggested that these clusters might be quasicrystalline regions surrounded by more freely dispersed liquid crystalline lipid molecules. Karnovsky and collaborators [8] formalized the concept of the organization of the lipid components of membranes into domains. However, it was not until the 1990's that membrane fractionation techniques improved sufficiently to provide the initial information on membrane micro-domains, and the concept of lipid rafts was proposed [9-14].

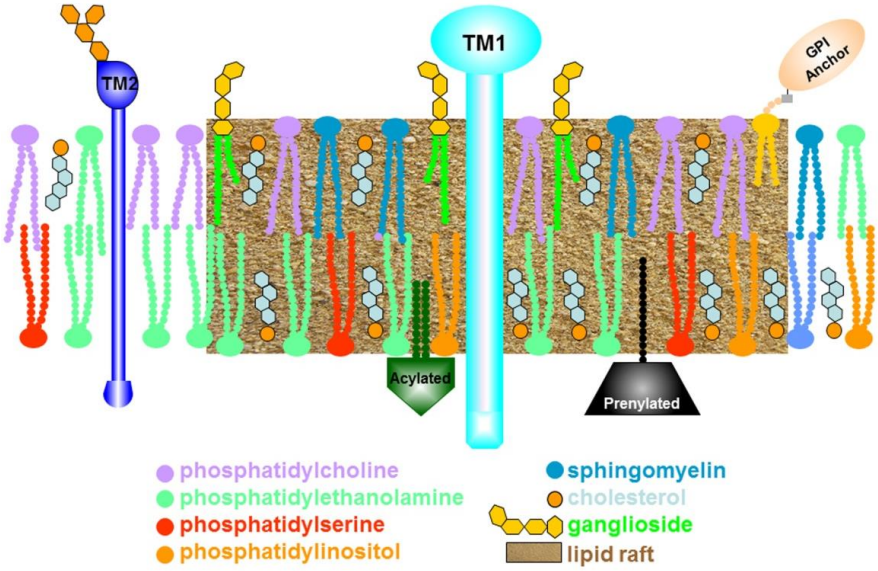

Figure 1. Schematic illustration of a lipid raft. Lipid rafts contain high levels of cholesterol, sphingolipids and gangliosides. GPI anchored proteins along with sphingomyelin and gangliosides are present in the outer leaflet. Acylated and prenylated proteins, phosphatidlyserine and phosphatidylethanolamine are located in the inner leaflet. Cholesterol is present in both leaflets and functions as a space filling molecule under the sphingolipid head groups. Transmembrane proteins may be associated with the lipid raft (TM1) or outside of the lipid raft (TM2).

The lipid rafts (Figure 1), present in all eukaryotic cells, are not a single stable structure in the plasma membrane. They are a dynamic and heterogeneous set of subdomains that differ in protein and lipid composition. Lipid rafts contain high levels of cholesterol (cholesterol levels in rafts are generally double those found in the plasma membranes from which they were derived), sphingolipids (such as sphingomyelin) and gangliosides [15-18]. Also, they are transitory in nature and their composition can change with the physiological state of the cell. The lipid rafts exhibit variability in their shape, size, lifetime and stability [19-21]. Lipid rafts are currently defined as dynamic ordered meso-scale (5-200 nm) assemblies of proteins and lipids of the plasma membrane and other intracellular membranes, such as Golgi membranes, that associate and dissociate on a sub-second timescale [5, 22-25].

A large number of studies suggest that lipid rafts selectively concentrate glycosylphosphatidylinositol (GPI)-anchored proteins on their outer side and proteins with lipid modifications (palmitoyl or myristoyl groups) and

cholesterol-binding proteins on the cytoplasmic side [26-30]. On the other hand, proteins with transmembrane segments have been shown to be targeted to lipid rafts by amino acid sequences in their extracellular [31], transmembrane [32], or intracellular domains [33]. However, little progress has been made in determining the nature of these protein-based raft targeting sequences, therefore it is difficult to predict, on the basis of sequence, whether a protein is likely to be directed to rafts [5]. Lipid raft composition, with a preponderance of longer saturated hydrocarbon chains that potentiate interdigitation between leaflets [34] and favors interaction with cholesterol [24], allows cholesterol to be tightly intercalated within the raft. The lipid rafts are highly organized and probably exist in a liquid-ordered (l_o) phase, different from the rest of the plasma membrane which consists mainly of phospholipids (with unsaturated tails) in a liquid-disordered (l_d) phase [35, 36]. The extent of packing depends on the degree of saturation of the lipid. The cis double bond present on unsaturated lipids introduces a rigid bend in the hydrocarbon tail which interferes with the tight packing and results in less stable aggregates [37]. These membrane micro-domains are characterized by a high melting temperature and resistance to solubilization in non-ionic detergents, such as Triton X-100, at low temperature [38]. They are dynamic in that they can coalesce or fragment into smaller units [30] and both proteins and lipids can move in and out of raft domains with different partitioning kinetics [29]. Lipid rafts can also form stabile platforms that are important in signaling, viral infection and membrane trafficking [23, 39, 40]. The high concentration of two types of the lipids, sphingolipids and cholesterol, confer stability on the lipid rafts formed in the plasma membrane [41]. Despite a body of evidence supporting the existence of raft domains, the raft concept is still being debated [42] and their true nature and even their existence in live cells has been a subject of controversy [5, 43-45]. These controversies concerning the existence of lipid rafts may be due to restrictions in the methods available to isolate and to characterize lipid rafts [46]. Lipid rafts have not been directly observed *in vivo* without substantial nonphysiological perturbation, such as long-term incubation in cold or extensive cross-linking of raft markers [20, 47]. Furthermore, the mechanisms that govern the associations among sphingolipid, cholesterol and specific membrane proteins in live cell membranes remain unclear [48]. The majority of the studies involving lipid rafts begin with detergent solubilization of whole cells followed by sucrose density gradient centrifugation and the recovery of detergent-resistant membranes from the light fractions of the gradient [49, 50]. However, the analysis of density gradient centrifugation experiments remains controversial

because there is an indication that detergents may force associations between components that are not associated in intact cells [51]. Fractionation results are also known to be severely altered by varying a number of factors such as the concentration of Triton X-100 [52, 53], the use of different detergents [54, 55] or the omission of detergents in general [56-58]. However, biochemical isolation of subcellular components remains an essential part of structure-function analysis in cells, even while recognizing that these procedures perturb cellular structure relative to those seen in live cells. Isolation of lipid rafts by detergent fractionation or other methods suffers the same pitfalls, but provides the means necessary for molecular characterization that can be further refined in live cell experiments [59]. Recently, compelling data has been presented that self-organization of lipids and proteins can induce a sub-compartmentalization that organizes the bioactivity of cell membranes [24, 25]. Lately, the lipid-based phase separation into liquid-ordered-like and liquid-disordered-like has been seen in giant plasma membrane vesicles (GPMVs) obtained by chemically induced blebbing in cultured cells [60, 61] or by using cell swelling to generate plasma membrane spheres (PMS) [62]. Johnson and collaborators, [63] using GPMVs showed that peripheral protein binding may be a regulator for lateral heterogeneity *in vivo*. These new approaches are very promising, allowing studies of the lipid domains in the absence of detergents. Advances in imaging and studies with improved integrated methodologies, such as flotation of detergent-resistant membranes, antibody-induced patching and immunofluorescence microscopy, immunoelectron microscopy, chemical crosslinking, single fluorophore tracking microscopy, photonic force microscopy, spectrofluorimetry, mass spectrometry, and fluorescence resonance energy transfer (FRET) are now providing insights into the existence and behavior of lipid rafts [3, 39, 47, 64-70]. Proteomic analyses have also provided confirmation of the raft localization of many proteins previously shown to partition into lipid rafts using other methods. These studies have also identified novel proteins in rafts and led to insights into the physiological regulation of rafts [5]. Lipidomic studies of lipid rafts have been done using rafts prepared by both detergent-free and detergent-containing protocols, when these results are compared, significant differences in lipid composition have been identified [15]. Thus, attention is required when the results of individual raft lipidomics studies are interpreted [5].

Studies of lipid rafts have also been complicated by imprecise nomenclature [39]. For example lipid rafts are also known as detergent-resistant membranes (DREMs) or glycosphingolipids-enriched membranes

(GEMs) [2, 14, 71], and caveolae were synonymous with lipid rafts for many years. In 1998, Harder *et al.*, using a cell system lacking caveolae, demonstrated that raft and non-raft markers segregated in the same cholesterol-dependent way in the absence of caveolae. These results showed that clustered raft markers segregate away from non-raft proteins in a cholesterol dependent, but caveolin independent manner [70]. Today caveolae are considered a subset of lipid rafts [3, 72]. Lipid rafts were so named because it was originally thought that they represented pre-existing domains in membranes into which different proteins partitioned [5].

Functional roles for lipid rafts and lipid heterogeneity in cellular processes have received increasing attention during the past decade [73]. These membrane micro-domains have been implicated in a variety of cellular processes, including pro- and anti-apoptotic events, host-pathogen interactions, endocytic events, such as viral entry, vesicle trafficking to or from the plasma membrane, signal transduction, cell adhesion, play roles in the initiation of many pharmacological agent-induced signaling pathways and toxicological effects [74-76]. However, the most important role of rafts at the cell surface may be their function in cell signaling by virtue of their affinity for signaling proteins, including transmembrane receptors, GPI anchored proteins, G proteins, RhoA, linker for activation of T cells (LAT, also used as a lipid raft marker) and Src kinases (Rous asian sarcoma tyrosine kinase) [42, 49]. The number of proteins reported to be regulated by specific lipid interaction is steadily increasing, but the precise structural mechanisms behind specific binding and receptor regulation in membranes remain uncharacterized [70]. Biochemical and genetic data have lent credence to the notion that rafts function as a specialized signaling platform in cell membranes [77-83]. Most likely, the function of the rafts is aided by stimulation-induced association and recruitment of various molecules with raft affinity, as well as varying degrees of raft engagement with the cytoskeleton [30, 71, 84]. Lipid rafts are also thought to be important sites for protein-tyrosine-kinase-mediated protein-protein interactions that are involved in the initiation of receptor signaling pathways [3, 71, 85]. It is well known that, in the case of tyrosine kinase receptors, adaptors, scaffolding proteins and enzymes are recruited to the cytoplasmic side of the plasma membrane as a result of ligand binding to form a signaling complex [86]. If receptor activation takes place in an ordered lipid raft, the signaling complex is protected from other proteins, such as membrane phosphatases, localized in the disordered region of the plasma membrane, that otherwise could affect the signaling process [44, 66, 87]. Lipid rafts serve as a platform for diverse signaling pathways, such as those mediated by growth

factors, morphogens, integrins [3] and antigen receptors on immune cells [40, 88-91].

This chapter presents evidence that lipid rafts play a pivotal role in signal transduction in mast cells and that the organization of various molecules in lipid rafts could modulate many biological processes in these cells.

Mast Cells

Mast cells were first recognized by Paul Ehrlich [92]. His microscopic observations revealed that mast cells had a high content of cytoplasmic granules, which stain metachromatically with basic aniline dyes such as toluidine blue (Figure 2).

Like blood cells, mast cells are derived from pluripotent bone marrow hematopoietic stem cells but, unlike other blood cells, which circulate in the blood, mature mast cells are not detected in circulation and reside in relatively low numbers within tissues, making isolation difficult [93]. Mast cells leave the bone marrow as progenitors and migrate to peripheral sites where they complete their differentiation under the influence of factors present at each site [94]. Mast cells normally reside in the connective tissue close to epithelia, associated with blood vessels, nerves, and are found in mucosa of the gastrointestinal, respiratory tracts, in the skin, near muscle cells and mucus-producing glands. In some species, including murine rodents, mast cells also occur within mesothelium-lined cavities, such as the peritoneal cavity [95, 96].

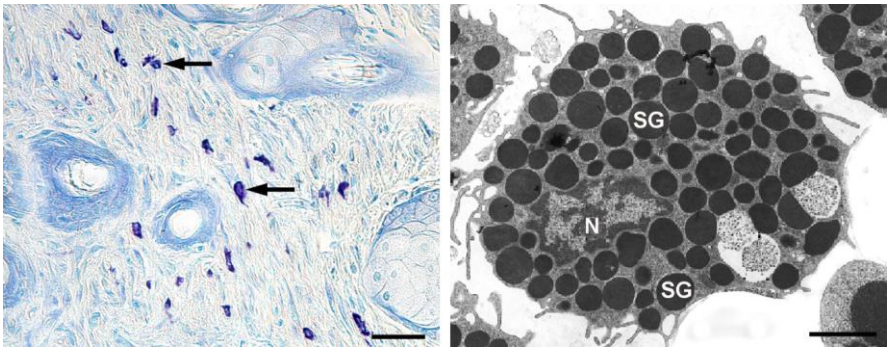

Figure 2. Mast cells in the dermis (arrows) stain metachromatically with toluidine blue. Bar equals 40µm. Transmission electron microscopy. Electron dense secretory granules can be seen in the cytoplasm in this mature peritoneal mast cell. Bar equals 5µm; N, nucleus; SG, secretory granule.

Kitamura and collaborators [97] characterized WBxC57 BL/6-FI-c-kit $^{w/Wv}$ mice (W/W^v mice), which are mast cell-deficient. Interestingly, an attractive feature of these mice was that their mast cell deficiency could be reconstituted by adoptive transfer of cultured mast cells. Furthermore, transfer of mast cells with targeted deletions in specific genes allowed the study of mast cell specific expression of particular proteins in a biological response. This model was a useful tool for investigations concerning the physiologic roles and the origin of mast cells [97]. In 2005, another mast cell-deficient mouse, W^{sh}/W^{sh}, was characterized [98]. Similar to W/W^v mice, the W^{sh}/W^{sh} mice also have mutations at the *W* locus, however, they are fertile and do not possess some of the c-kit-mediated developmental defects such as anemia, although they do have an age-dependent loss of mast cells that is complete by about 12 weeks of age. Studies using both mouse mast cell deficient models have contributed to a better knowledge of mast cell biology and have revealed that mast cells are multifaceted immune cells with diverse roles in health and disease [93, 99].

Mast cells have long been known to play a critical role in inflammatory and allergic reactions [95, 96, 100-107]. Apart from their roles in pathological conditions, the biological functions of mast cells include roles in both innate and adaptive immunity, involvement in host defense mechanisms against parasites, immunomodulation of the immune system, tissue repair and angiogenesis [100, 101, 108-111]. Recently, mast cells have gained new importance as immunoregulatory cells with the discovery that they are a major source of cytokines and chemokines [93, 96, 112, 113].

Mast cells have a large repertoire of cell-surface receptors, such as Toll-like receptors (TLRs), the high-affinity receptor for immunoglobulin E (FcεRI) and the high-affinity receptor for immunoglobulin G (FcγRI), enabling them to interact both directly and indirectly with pathogens and environmental toxins. Activated mast cells possess the ability to produce and release a wide variety of mediators, which serve to activate the immune response or to amplify an existing response [93].

Although mast cells may be activated by a number of stimuli and pathways [114, 115], the major mechanism of activation and subsequent degranulation is through the FcεRI, present in the plasma membrane of mast cells, epidermal Langerhans cells, eosinophils, and basophils [116]. FcεRI is expressed as a heterotetrameric structure composed of one α subunit with an extracellular domain that binds immunoglobulin E (IgE), a four transmembrane-spanning β subunit, and two identical disulphide linked γ subunits [3, 117, 118]. The carboxyl terminal cytoplasmic domains of both β

and γ subunits contain an immunoreceptor tyrosine-based activation motif (ITAM), common to all multi-subunit immune recognition receptors, that is critical for cell activation. Mast cell activation is initiated by the binding of oligomeric antigens to receptor-bound IgE, which crosslinks FcεRI and results in its aggregation.

The first recognized biochemical event of the cytoplasmic signal transduction cascade, after crosslinking of two or more FcεRI, involves phosphorylation, usually by Lyn, a member of the Src family of tyrosine kinases, of two conserved tyrosine residues within the ITAMs of both β and γ subunits of the receptor. The tyrosine-phosphorylated ITAMs create a novel binding surface that is recognized by additional cytoplasmic signaling molecules, such as the protein tyrosine kinase Syk which binds mainly to the γ subunit, via its tandem Src homology 2 (SH2) domains. This interaction results in an increased kinase activity that rapidly shifts the equilibrium of the cell from a resting state to an activated state. This Syk mediated signal amplification results in a direct or indirect activation of several proteins, LAT, Vav, phospholipase C- γ 1 (PLC- γ1) and PLC- γ2. Finally, downstream activation leads to calcium (Ca^{2+}) release from the endoplasmic reticulum (ER), which is followed by Ca^{2+} influx from outside of cell, causing an increase in the cytosolic free Ca^{2+} concentration [119], activation of other enzymes and adaptors, and rearrangement of the cytoskeleton that culminates in the release of three classes of mediators: (1) *preformed mediators* (stored in secretory granules), such as histamine, heparin, β-hexosaminidase, neutral proteases, acid hydrolases, major basic protein, carboxypeptidases, and some cytokines and growth factors; (2) *newly formed lipid mediators*, such as prostaglandins and leukotrienes; and (3) *newly synthesized mediators*, that include growth factors, cytokines and chemokines [104, 117, 120].

Accumulating evidence suggests that lipid rafts or raft components play a pivotal role in signal transduction via FcεRI in mast cells and that the organization of various molecules in lipid rafts could modulate many biological processes in these cells.

Lipid Rafts, Mast Cells and Signal Transduction

The first signaling complex convincingly shown to involve lipid rafts was IgE during an allergic immune response [88, 121]. IgE signaling was initially

thought to be based on protein-protein interactions alone, but several observations indicated that lipid rafts, are involved in this process [20, 88, 122-127]. The first hint of lipid raft involvement came from the finding that FcεRI is soluble in Triton X-100 at steady state, but after crosslinking becomes insoluble in low concentrations of this detergent [88]. Moreover, in unstimulated cells, FcεRI is dispersed throughout the plasma membrane, but upon activation rapidly aggregates [128, 129] and can then be found on the cell surface in association with the ganglioside GM_1 [130-133] and the glycosylphosphatidylinositol-linked protein Thy-1 [134]. Fridriksson and collaborators [135], using Fourier transform mass spectrometry (FTMS) with electrospray ionization mass spectrometry (ESI/MS) compared the phospholipid composition of lipid rafts with plasma membrane vesicles and whole cell lipid extracts from RBL-2H3 cells, a rat mast cell line, and observed that antigen stimulation of FcεRI causes a significant change in the phospholipid composition of lipid rafts, with a substantial increase in the ratio of polyunsaturated to saturated plus monounsaturated phospholipids.

Despite numerous studies on mast cell activation through FcεRI, the detailed mechanism by which cross-linking promotes the initial phosphorylation by Lyn and how and/or where these proteins interact remains unclear [14, 20, 87, 136]. Davey and collaborators [20] suggested that protein-protein interaction (IgE-FcεRI cross-linking) recruits essential signaling proteins and lipid molecules into more ordered domains that serve as a platform for signaling. The structural basis for the association of FcεRI with lipid rafts is partially understood. Both β and γ subunits are palmitoylated which could facilitate their association with lipid rafts [137]. However, Field and collaborators [52] investigated the structural basis for the interaction of FcεRI with isolated lipid rafts and observed that this interaction is not mediated by the β subunit or by the cytoplasmic tail of the γ subunit of the receptor. In this study, the results point to the transmembrane segments of α or γ subunits as being primarily responsible for aggregation-dependent association of FcεRI with lipid rafts. However, more extensive analyses are required to completely define the structural basis for FcεRI and lipid raft association.

An approach extensively used to better understand the role of lipid rafts in FcεRI-mediated signaling has been the study and/or the manipulation of the lipid constituents of rafts, such as cholesterol and gangliosides [3, 138, 139] or electron microscopy studies [66]. Lipid rafts present three to five times more cholesterol than the rest of the plasma membrane, which presumably occurs because the cholesterol has higher affinity for raft sphingolipids than for

unsaturated phospholipids. Cholesterol is thought to play role as a spacer between the hydrocarbon chains of the sphingolipids and function as dynamic glue that keeps the raft assembly together [3]. Removal of raft cholesterol leads to dissociation of most proteins from rafts and renders them nonfunctional [140].

Methyl-β-cyclodextrin (MβCD), a carbohydrate molecule with a pocket for binding cholesterol, has been extensively used to deplete the plasma membrane of cholesterol and subsequently disrupt lipid rafts [3]. Also, MβCD has been used to study the role of lipid rafts in FcεRI-mediated signaling, particularly in early events of the signal transduction such as tyrosine phosphorylation of FcεRI by Lyn [138]. Sheets and collaborators [121] demonstrated that phosphorylation of FcεRI occurs in a cholesterol-dependent manner and that cholesterol depletion reduces tyrosine phosphorylation of FcεRI after cross-linking. In parallel to its inhibition of tyrosine phosphorylation, cholesterol depletion disrupts the interactions of aggregated FcεRI and Lyn in intact cells. Cholesterol repletion restores receptor tyrosine phosphorylation together with the structural interactions among the raft constitutes, providing strong evidence that lipid raft structure, maintained by cholesterol, plays a critical role in the initiation of FcεRI signaling. Cholesterol depletion by MβCD in RBL-2H3 cells also reduced the release of β-hexosaminidase activity in cells stimulated via FcεRI [139, 141-143]. These data suggest that cholesterol depletion by MβCD affects IgE signaling due to the disruption of lipid rafts resulting in a failure to form a signaling complex. Additionally, Young and collaborators [87] provided evidence that when Lyn is segregated in lipid rafts it has substantially higher kinase activity than Lyn outside the membrane micro-domains. These data suggest that some undefined components of lipid rafts may influence the kinase activity of Lyn [138] and consequently FcεRI signal transduction.

Another constituent of lipid rafts, f flotillin-1, was initially identified as a caveolae-associated membrane protein and is a marker protein of lipid rafts although its physiolological role is still not clear [144, 145]. Kato and collaborators [138] using flotillin-1 knockdown RBL-2H3 cells showed that flotillin-1 regulates the kinase activity of Lyn in mast cells. In the flotillin-1 knockdown cells there was a significant decrease in Ca^{2+} mobilization, the phosphorylation of ERKs, tyrosine phosphorylation of the γ-subunit of FcεRI, and IgE-mediated degranulation. This study also showed that flotillin-1 is constitutively associated with Lyn in lipid rafts in RBL-2H3 cells, and that antigen stimulation induced an increase in flotillin-1 binding to Lyn, resulting in an enhancement of Lyn kinase activity. These data suggest that flotillin-1 is

an important component of FcεRI-mediated mast cell activation, and regulates the kinase activity of Lyn in lipid rafts.

Gangliosides are structurally important for lipid raft assembly and function. The rigid structural nature of the ceramide anchor in gangliosides, coupled with the ability of sphingolipids to associate with cholesterol, is thought to drive the assembly of lipid rafts [3, 146]. Two unique gangliosides (α-galactosyl derivatives of the ganglioside GD_{1b} antigens I and II) are present on the surface of rodent mast cells [147-150] and are specifically recognized by the monoclonal antibody (mAb) AA4. Previous studies using mAb AA4 [148, 149] demonstrated that the α-galactosyl derivatives of the ganglioside GD_{1b} were present only in mast cells and not in any other cell type in all 19 rat [149] and 18 mouse [150] tissues examined, which confirms the specificity of mAb AA4 for rodent mast cells. These α-galactosyl derivatives of the ganglioside GD_{1b}, antigens I and II, contain respectively one and two additional α-galactosyl residues when compared with GD_{1b}. The gangliosides derived from GD_{1b} have been identified as components of lipid rafts in the plasma membrane of RBL-2H3 cells [120, 121, 139]. They also seem to be intimately involved with signaling through FcεRI. Although the functional role of these gangliosides is not clear, previous studies have shown that when the α-galactosyl derivatives of ganglioside GD_{1b} were bound to mAb AA4, histamine release was inhibited in a time and concentration dependent manner [149, 151]. Binding of mAb AA4 to RBL-2H3 cells resulted in an increase in intracellular calcium, phosphatidylinositol hydrolysis, and a redistribution of PKC. However, the magnitude of these changes was less than those after FcεRI cross-linking, and unlike FcεRI activation, these changes were not accompanied by histamine release [149]. These derivatives of the ganglioside GD_{1b} co-precipitated with the Lyn. Although mAb AA4 binds to sites close to FcεRI the association between Lyn and these gangliosides was independent of the association of the gangliosides with FcεRI. Moreover, the association of Lyn with the gangliosides is much stronger than the association of Lyn with FcεRI. These associations suggest that a complex that includes gangliosides, FcεRI and Lyn is essential for modulation of signal transduction in mast cells [149, 151-154].

Furthermore, analysis of the subcellular distribution by sucrose gradients showed that following FcεRI activation there was a shift in the distribution of the gangliosides and FcεRI to the lipid raft fractions [120, 139]. The movement of these gangliosides and of the FcεRI into the lipid rafts may be another mechanism that regulates signal transduction in mast cells by facilitating formation of a signaling complex. In addition, when FcεRI is

crosslinked, the raft components patch selectively into restricted cell surface domains [134]. Non-raft proteins are not recruited into these domains [57]. Harder et al. also showed that this co-redistribution of raft components is dependent on cholesterol. These raft domains can selectively incorporate or exclude proteins, and thereby control protein–protein and protein–lipid interactions.

In 2008, Silveira e Souza and collaborators [139], using a cell line deficient in the α-galactosyl derivatives of the ganglioside GD_{1b}, as well as the parent cell line, RBL-2H3, demonstrated the importance of these gangliosides for lipid raft organization and consequently for FcεRI-mediated degranulation in rodent mast cells. In this study the authors observed an increased inhibition in the release of β-hexosaminidase activity in the mutant cell line after FcεRI stimulation, but not after exposure to calcium ionophore. Moreover, treatment of RBL-2H3 cells with compounds that inhibit ganglioside synthesis also reduced release of β-hexosaminidase activity showing that release of β-hexosaminidase activity is dependent on the presence of gangliosides.

In addition to lipid raft assembly, another possible role for the mast cell specific gangliosides in signal transduction may be to facilitate the association between Lyn and FcεRI. Because FcεRI itself has no intrinsic kinase activity, the tyrosine phosphorylation induced by receptor cross-linking is a secondary event that occurs after aggregation of FcεRI and its movement into lipid rafts [154]. Therefore, formation of lipid raft complexes that include gangliosides, and associated proteins, such as Lyn, LAT, flotillin-1 and FcεRI, have an important role in receptor-mediated signal transduction.

In addition to gangliosides, sphingomyelin, a type of sphingolipid also has a role in signaling events in mast cells. The sphingomyelin pathways with its products ceramide, sphingosine (S) and S-1-phosphate (S1P) are known to play roles in signal transduction [155, 156]. Sphingosine kinase has been recognized as an essential signaling molecule that mediates the intracellular conversion of S to S1P. In mast cells, induction of sphingosine kinase and generation of S1P have been linked to the initial rise in Ca^{2+}, released from internal stores, and to degranulation. Urtz and collaborators [157] showed that sphingosine kinase type 1 (SPHK1) interacts directly with Lyn and this interactions leads to the recruitment of this lipid kinase to the FcεRI. The interaction of SPHK1 with Lyn caused enhanced lipid and tyrosine kinase activity. After FcεRI triggering, enhanced sphingosine kinase activity was associated with FcεRI in lipid rafts in mast cells.

Mast cells also produce structures such as cytonemes or tunneling nanotubes used for intercellular communication and this intercellular

communication may be important during allergic and inflammatory responses following co-stimulation of FcεRI and CCR1 (chemokine receptor 1) [99]. Albeit the process of cytoneme formation remains poorly understood, the fact that cholesterol depletion reduced the formation of cytonemes, suggests that lipid rafts may participate in cytoneme formation in mast cells, either by promoting membrane integrity or by participating in cell signaling.

Studies have shown that establishing and maintaining lipid rafts are necessary for many biological processes besides cell signaling [158, 159]. In mast cells, the structure-function relationship of lipid rafts or of rafts constitutes, are important in various biological events.

Lipid Rafts and Mast Cells: Morphology, Endocytosis and Cell Development and Recruitment

Morphology

Using the parent cell line, RBL-2H3, [100, 149, 160-162], and a ganglioside deficient cell line, D1, the influence of lipid rafts on cell structure and organization has been examined [163]. The D1 cell line is deficient in GM_1 gangliosides and in the α-galactosyl derivatives of the ganglioside GD_{1b}.

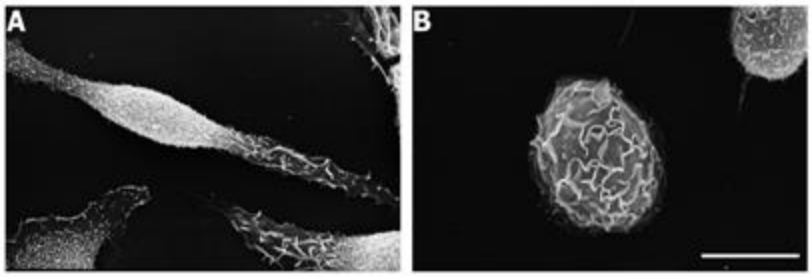

Figure 3. Ganglioside-deficient D1 cells have an altered morphology. By scanning electron microscopy, RBL-2H3 cells (A) are spindle shaped and their surface is covered with short microvilli. In contrast, D1 cells (B) are rounded and their surface is covered with large membrane ruffles. Both cell lines were stimulated for 5 minutes via FcεRI. Bar equals 10μm.

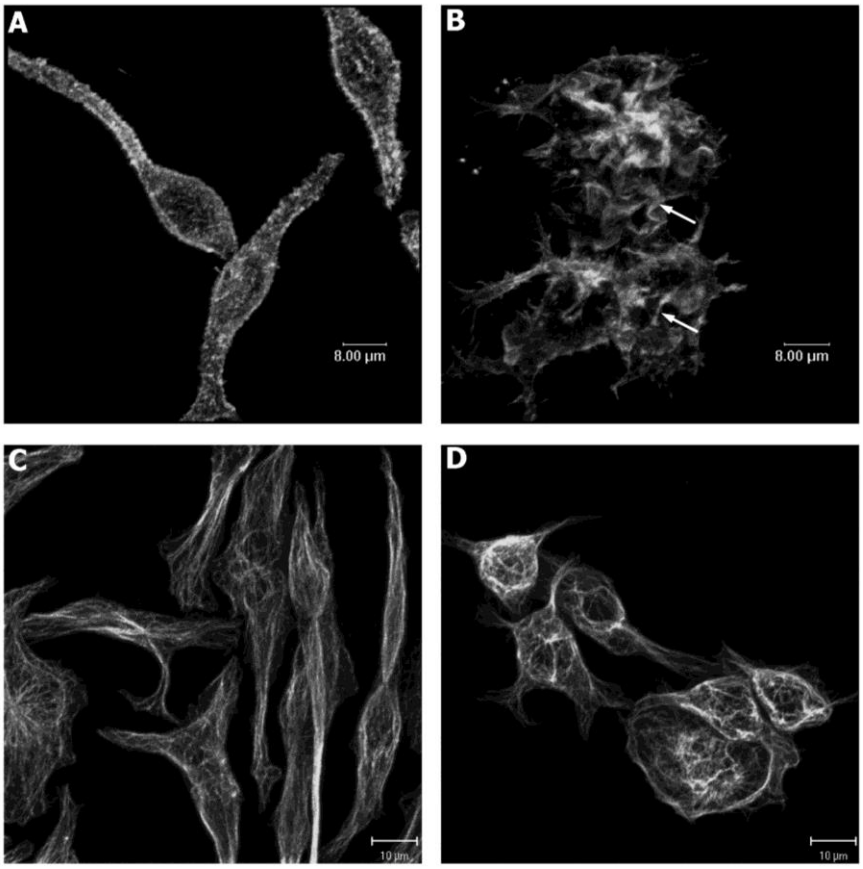

Figure 4. The cytoskeleton is altered in D1 cells. The F-actin distribution in RBL-2H3 and D1 cells reflects their morphology. Actin filaments in RBL-2H3 cells (A) lie under the plasma membrane following the spindle shape of the cells and in association with microvilli. In D1 cells (B), actin filaments are concentrated in large membrane ruffles (arrows) and extend into the cytoplasmic projections. F-actin was labeled with phaloidin-Alexa 488. In RBL-2H3 cells (C), microtubules are organized into bundles that followed the fusiform shape of the cells, whereas in D1 cells (D), microtubules appear as thick bundles that are restricted to the cell body and are not present in the cytoplasmic extensions. To visualize the microtubules, the cells were fixed, permeabilized and labeled with anti-β-tubulin antibody and subsequently with a secondary antibody conjugated to Alexa-488. Samples were examined using a Leica TCS-NT laser scanning confocal microscope.

The mutant cell line D1 has a cellular morphology which is distinct from RBL-2H3 cells. RBL-2H3 are spindle shaped and their surface is covered with short microvilli, while D1 cells are rounded and their surface is covered with

large membrane ruffles (Figure 3), suggesting that the gangliosides are important in the maintenance of normal cell morphology.

The morphological changes observed in D1 cells may be related to the lipid composition of these cells. This cell line presents a large decrease in glycosphingolipids, such as GM_1 and the α-galactosyl derivatives of the ganglioside GD_{1b}, which may affect many physicochemical properties of the plasma membrane. According to Kato [142] the lipid composition can influence membrane stability, membrane fluidity, lipid packing, bilayer curvature, and hydration elasticity, as well as anchorage of the cytoskeleton to the plasma membrane. In fact, D1 cells show an abnormal distribution of actin filaments and microtubules (Figure 4) [163]. A growing body of evidence indicates that lipid rafts are essential for membrane-cytoskeleton coupling and the association of Lyn and other raft markers with crosslinked FcεRI is regulated by interactions with F-actin [5, 134, 164]. It is possible that in the D1 mutant cells, the disorganization of both lipid rafts and actin filaments contributes to impaired degranulation after FcεRI stimulation [139, 163]. Furthermore, the actin cytoskeleton is known to participate in regulating and activating raft-associated signaling events [165-167].

The factors that govern the formation of lipid rafts continue to be elucidated, but lipid raft formation requires actin polymerization. The connection of lipid raft proteins with actin filaments directs the lateral distribution and mobility of these membrane proteins [77, 168]. The extent to which the actin cytoskeleton participates in the formation of membrane rafts is not yet established. Han [59] observed, with mass spectrometric analyses, that inhibitors of actin polymerization (cytochalasin D and latrunculin A) cause a similar, but faster, change in phospholipid composition of lipid rafts than activation through FcεRI. Thus, perturbations in the actin filaments affect the organization of lipid rafts in RBL-2H3 cells. The changes in raft phospholipid composition following stimulation depend on actin polymerization and appear largely independent of the tyrosine phosphorylation cascade that is activated in parallel to these cytoskeletal changes.

The actin cytoskeleton is a dynamic structure that changes in response to extracellular signals, and therefore represents one mechanism governing the establishment and distribution of lipid rafts in the plasma membrane [169]. Lipid rafts may be structured by a synergistic interaction between the cortical actin filaments and the lipid rafts themselves, and that many of the structural and functional properties of rafts require an intact actin cytoskeleton [170]. An important regulator of membrane-cytoskeleton interactions is the phosphoinositide PIP2, which is a minor lipid component of the plasma

membrane that is known to regulate the organization of the actin cytoskeleton and in particular the formation of actin-membrane linkages [171]. PIP2 also serves as a co-factor for many of the proteins that anchor actin filaments to the plasma membrane [171, 172]. Protein binding to PIP2 often occurs through a PIP2-specific recognition sequence, in many cases represented by a PIP2-specific pleckstrin homology (PH) domain [173-175]. Some actin binding proteins (ABPs) are thought to link actin filaments and PIP2-enriched rafts. Gelsolin is one of the ABPs present in lipid rafts [176].

Microtubules are another of the major determinants of cell shape and polarity [177, 178]. In the ganglioside deficient D1 cells the arrangement of microtubules was completely disorganized demonstrating that the abnormal morphology observed in the mutant cell line could be correlated with the decrease in gangliosides that leads to lipid raft disorganization [163]. Therefore, gangliosides are structurally important for lipid raft assembly and function and are critical for maintenance of mast cell morphology.

Endocytosis

Concomitant with the concept of lipid rafts and the lateral movement of proteins in the plasma membrane, it was observed that plasma membrane proteins could suffer a selective reorganization followed by internalization of these proteins [179-181]. Receptor mediated endocytosis, including endocytosis of FcεRI, is a temporally and spatially organized process [120, 182]. Receptor-induced endocytosis falls into to two main categories, clathrin-dependent and clathrin-independent endocytosis. In general both types of receptor mediated endocytosis are dependent on lipid rafts. Clathrin-dependent endocytosis is the best-studied of the two. This pathway requires the presence of the protein clathrin, which is the major component of the endocytic vesicle coat [183-185]. Cargo associated adapters recruit clathrin to the plasma membrane and promotes its assembly into a polyhedral lattice leading to the formation of a clathrin-coated vesicle. The major adapter for clathrin is the adapter protein complex-2 (AP-2) [200]. Clathrin-independent endocytosis is not as well understood. The major form of clathrin-independent endocytosis is dependent on lipid rafts that contain the protein caveolin. Caveolin induces the formation of caveolae at the cell surface [186, 187]. The formation of a bud followed by pinching-off of the vesicles, to into of cytoplasm, requires the activity of the GTPase dynamin [184, 188]. When endocytosis is lipid raft-dependent, internalization of the receptor is sensitive to cholesterol depletion

and the internalized receptor colocalizes with lipid raft components including flotillin and gangliosides such as GM_1 and GD_{1b} [120, 189].

In mast cells, after activation, crosslinked FcεRI is endocytosed through clathrin-coated vesicles and transported by the endosomal system for eventual degradation in lysosomes or dissociation from the ligands [190-192]. Thus, clathrin-dependent endocytosis has long been thought to be a mechanism for the attenuation of receptor-activated responses [119]. In unstimulated mast cells, FcεRIs are dispersed throughout the plasma membrane but upon activation the receptors rapidly aggregate and can be found on the cell surface in lipid rafts in association with GM_1 [129, 133], the α-galactosyl derivatives of the ganglioside GD_{1b}, protein tyrosine kinase Lyn, and LAT [52, 120, 121]. However, only when the mast cells are activated via FcεRI does a significant internalization of the GD_{1b} derivatives occur [120, 193]. Oliver and collaborators [120] showed that upon activation of FcεRI, the gangliosides derived from GD_{1b} are internalized together with the receptor, following the same pathway to lysosomes. In order to verify the importance of the gangliosides derived from GD_{1b}, present in lipid rafts, in endocytosis Mazucato and collaborators [193] used mutant RBL-2H3 cells that were deficient in these gangliosides. The ganglioside-deficient mast cells showed a delay in the internalization of FcεRI into the endocytic compartments, indicating that the gangliosides derived from GD_{1b} are essential for the endocytosis of FcεRI. The endocytic process itself may play an important role in signal transduction [182, 186, 194] and may facilitate the structural preservation of signaling complexes, and the prolongation of the signal since these gangliosides as well as the FcεRI are associated in lipid rafts and remain associated during endocytosis.

In view of the importance of lipid raft integrity for efficient receptor endocytosis, it has been observed that the FcεRI ubiquitination is a key mechanism for the regulation and control of antigen-dependent endocytosis of receptor complexes [189]. Moreover, it has been demonstrated that ubiquitin ligases Cbl and Nedd4 are recruited into lipid rafts upon IgE triggered cell signaling [195]. Nedd4 is known to ubiquitinate membrane receptors [196]. The ubiquitin Cbl is a good candidate to mediate FcεRI ubiquitination since it participates in various functions such as cis-and-trans-ubiquitination [197]. It is phosphorylated upon FcεRI engagement [198] and negatively regulates Syk kinase [199]. Molfetta and collaborators [189, 200] suggested that the recruitment of engaged FcεRI subunits into lipid rafts precedes their ubiquitination, and that integrity of lipid rafts is required for receptor

ubiquitination and endocytosis, contributing to the down-regulation of FcεRI-mediated signaling.

Mast Cell Maturation

The expression of lipid raft components, such as the α-galactosyl derivatives of the ganglioside GD_{1b}, on the mast cell surface also appears to be related to mast cell maturation. Previous studies using mAb AA4 showed that the α-galactosyl derivatives of the ganglioside GD_{1b} were present only in mast cells and not in any other cell type in all rat tissues examined [148, 149]. However, in bone marrow, a population of large, poorly differentiated cells, presumably immature mast cells also stained with mAb AA4 [149]. Later these cells were indeed shown to be very immature and immature mast cells [201, 202]. Since the heterogeneity of the maturing mast cells makes them impossible to separate from other cells on the basis of their density and mAb AA4 binds only to cells which can be identified as mast cells [201, 203], the gangliosides recognized by mAb AA4 may be considered a powerful marker for rodent mast cells. On the other hand, undifferentiated mast cell precursors in the bone marrow do not express, the α-galactosyl derivatives of the ganglioside GD_{1b} recognized by mAb AA4. Jamur and collaborators [150] isolated and characterized a committed mast cell precursor in the bone marrow of adult Balb/c mice, by immunoaffinity selection using 2 monoclonal antibodies, mAb AA4 and mAb BGD6. While mAb AA4 recognizes 2 derivatives of the ganglioside GD_{1b} that are unique to the surface of rodent mast cells [147-149], mAb BGD6 binds to the surface of RBL-2H3 mast cells at sites unrelated to FcεRI. Both mAb AA4 and mAb BGD6 bind to granulated mast cells in all stages of maturation in the bone marrow as well as binding to a very immature cell that is not recognized by mAb AA4. This mast cell precursor was characterized as BGD6+, $CD34^+$, $CD13^+$, c-kit^+, $FcεRI^-$ and $AA4^-$ (negative for the expression of derivatives of the ganglioside GD_{1b}). These gangliosides begin to be expressed on the cell surface jointly with FcεRI and at the same time as the initiation of the formation of cytoplasmic granules in very immature mast cells and continue to be expressed by mast cells in all stages of maturation [150]. These data suggest that mast cell lipid rafts or raft constitutes are related to mast cell maturation and function.

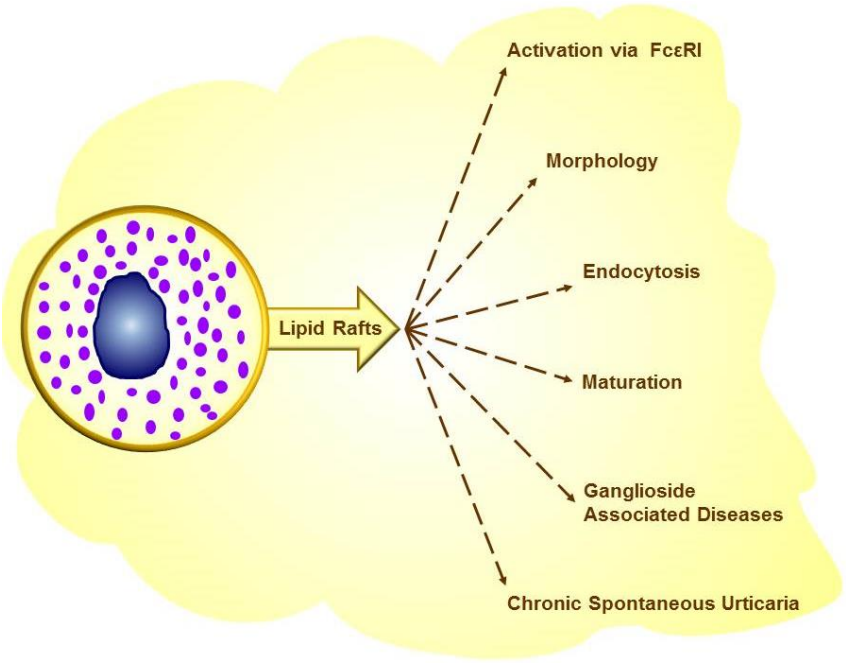

Figure 5. The diagram represents the involvement of lipid rafts in various aspects of mast cell biology. Lipid rafts are important in the modulation of many cellular processes such as activation through FcεRI, maintenance of cell structure, endocytosis, maturation, and are relevant in diseases related to gangliosides.

Conclusion

Although several aspects of lipid raft structure and function in mast cells still remain to be elucidated, this chapter presents evidence that, undoubtedly, lipid rafts and their constitutes modulate many of biological processes in mast cells (Figure 5). Further research to better define the role of lipid rafts in mast cells could offer novel targets for immunotherapies and treatment of diseases in which mast cells and/or their mediators are involved.

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